Immunohistochemical Alterations in Basement Membrane Components of Squamous Cell Carcinoma

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To investigate alterations in the basement membrane (BM) in squamous cell carcinoma (SCC), we investigated 20 tumors. Four had the cytologic characteristics of Bowen's disease (SCC-BD) and 16 did not have them (SCC-NB). Tumors were studied immunohistochemically by double immunofluorescent staining by using mouse monoclonal antibodies to the core protein of heparan sulfate proteoglycan (HSPG) and chondroitin 6-sulfate glycosaminoglycan (Ch6S) as well as rabbit antiserum to laminin (LN) and type IV collagen (C-4). In well-differentiated and highly keratinized SCC-NB, LN, C-4, and HSPG could be detected in the tumor nest BM and showed no loss of continuity, but they were largely lost in poorly differentiated and poorly keratinized SCC-NB. This suggests that poorly differentiated SCC-NB cause greater enzymatic degradation of BM components than well-differentiated SCC-NB. Ch6S was detected in parts of the BM of SCC-BD, but it was absent in all SCC-NB examined. It appears that SCC-NB have lost the ability to synthesize Ch6S, and that SCC-BD degrade Ch6S although they continue to produce it. Thus, it appears that in SCC the BM is qualitatively different from that of normal epidermis, and that SCC-BD can be distinguished from SCC-NB by the Ch6S content of the BM. *J Invest Dermatol 96:250–254, 1991*

During invasion and metastasis, epithelial tumor cells have to traverse various tissue compartments [1–3]. Because the basement membrane (BM) provides a mechanical barrier against tumor invasion, its destruction represents the first step in the invasion and metastasis by epithelial tumors [1–3]. Alterations of BM components in squamous cell carcinoma (SCC), such as changes in type IV collagen (C-4) [4–7] and laminin (LN) [4], have been extensively investigated. However, the results of these studies are contradictory; one group of investigators claims that loss of the BM is associated with invasive SCC [4], whereas another group does not [5–7]. In addition, the relationship between the extent of BM loss and metastasis or tumor histology has not been investigated sufficiently in these reports.

Several extracellular matrix components, including heparan sulfate proteoglycan (HSPG) [8–10] and chondroitin 6-sulfate glycosaminoglycan (Ch6S) [11,12], have been demonstrated immunohistochemically in the BM at the dermo-epidermal junction (DEJ). HSPG apparently plays an important role in the deposition of the other BM components, and their distribution is altered in transformed cells [13]. Thus, it appears that they may influence the behavior of tumor cells. Defective expression or the absence of Ch6S has been demonstrated in the epidermal BM of individuals with both dominant and recessive dystrophic epidermolysis bullosa, and such changes have been suggested to have a possible pathogenic role in these disorders [12]. Although cell-surface glycosaminoglycan (GAG) on some tumors have been demonstrated to be biochemically altered [14], there is little information available about alterations of these proteoglycans in the BM of malignant tumors.

In the present research, we studied the changes in C-4, LN, and proteoglycans in the BM with SCC and the relationship between the extent of the changes in BM components and the histopathology.

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Abbreviations:

BM: basement membrane
Ch6S: chondroitin 6-sulfate glycosaminoglycan
C-4: type IV collagen
DEJ: dermo-epidermal junction
GAG: glycosaminoglycan
HS: heparan sulfate glycosaminoglycan
HSPG: heparan sulfate proteoglycan
LN: laminin
MoAb: monoclonal antibody
PBS: phosphate-buffered saline
SCC: squamous cell carcinoma
SCC-BD: squamous cell carcinoma that has cytologic characteristics of Bowen's disease
SCC-NB: squamous cell carcinoma that does not have cytologic characteristics of Bowen's disease

MATERIALS AND METHODS

Sources of Antibodies and Enzyme LN was purified from Engelbreth-Holm-Swarm tumor by the method of Timpl et al [15]. C-4 was purified from human placenta by the method of Glanville et al [16], and the preparation was passed through a Sepharose 4B column coupled with anti-LN immunoglobulin to remove any contaminating LN. The identification and purity of these antigens was established from their Coomassie Blue staining pattern after electrophoresis on 4.5% polyacrylamide disc gels containing sodium dodecyl sulfate.

New Zealand white rabbits were immunized intramuscularly in the back with LN or C-4 (total 0.25 mg/ml), and emulsified with an equal volume of Freund's complete adjuvant. Every 2 weeks the same amounts of these antigens emulsified with adjuvant were injected in the same way, and the rabbits were bled.

A monoclonal antibody (MoAb), HS47, was obtained from the

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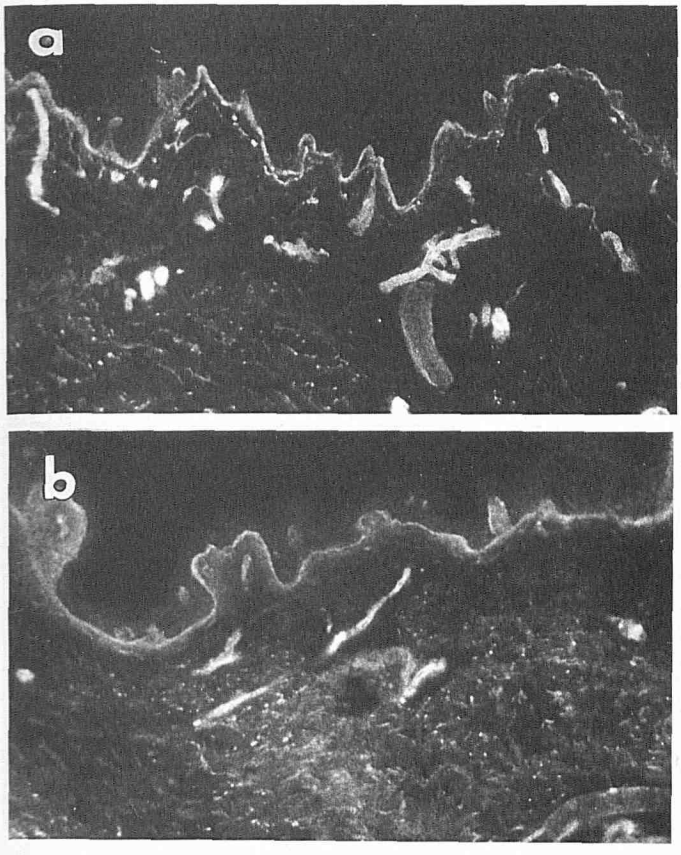

Figure 1. Immunofluorescent staining of normal human skin with anti-HSPG and anti-Ch6S antibody. HSPG (*a*) and Ch6S (*b*) are linearly observed at the DEJ and in the BM of dermal blood vessels. (Magnification ×100.)

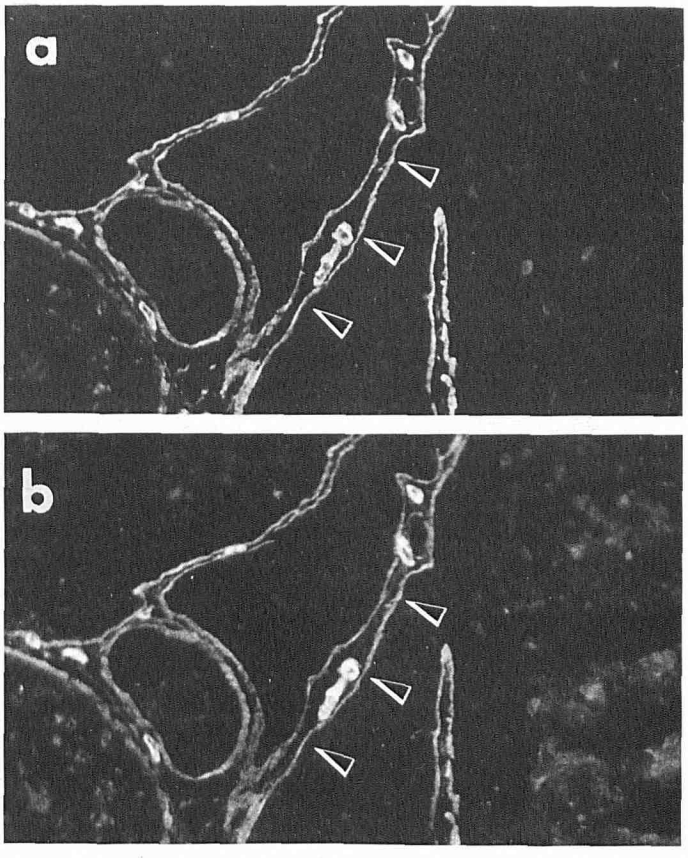

Figure 2. Double immunofluorescent staining of well-differentiated SCC-NB with anti-LN and anti-HSPG antibodies. LN (*a*) and HSPG (*b*) are located in the BM of both tumor nests and capillaries and show no loss of continuity. *Arrowheads,* the BM of the tumor nest. (Magnification ×100.)

hybridization of spleen cells from a mouse immunized with purified HSPG and NS-1 myeloma cells, as described previously [8]. MoAb 3B3, which recognizes Ch6S, has been fully documented by Fine and Couchman [11,12], and was purchased from Seikagaku-Kogyo Co. Ltd. (Tokyo, Japan). The MoAb, HK-1 [17], was kindly provided by Dr. Hashimoto of the Department of Dermatology at Wayne State University, Detroit, USA.

Rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated rabbit anti-mouse immunoglobulins were obtained from Tago Inc. (Burlingame, CA) and Dakopatts a/s (Glostrup, Denmark), respectively.

Chondroitinase ABC (from *Proteus vulgaris*) was purchased from Seikagaku-Kogyo.

Tumor Specimens We examined four SCC that had the cytologic characteristics of Bowen's disease (SCC-BD) and 16 other SCC that did not have those characteristics (SCC-NB). Specimens were obtained from biopsied or resected tumors, embedded in OCT compound, and quickly frozen in liquid nitrogen. Sections were cut at a thickness of 6 μm on a cryostat and dried in room air for 30 min.

Procedures After being washed in phosphate-buffered saline (PBS) (pH 7.4), the sections were incubated with rabbit antibodies to C-4 or LN, rinsed in PBS, then incubated in rhodamine-conjugated goat anti-rabbit IgG. After rinsing, some of these stained

Table I. Alterations of BM Components in SCC

Histology	LN and C-4	HSPG	Ch6S	Number of Cases[a]
SCC-NB	Continuous	Continuous	Absent	8
	Disrupted	Disrupted	Absent	8
SCC-BD	Continuous	Continuous	Disrupted[b]	1
	Disrupted	Disrupted	Disrupted[b]	3

[a] SCC were divided into four groups by the pattern of findings on immunohistochemistry with anti-BM components antibodies.
[b] All SCC in which the BM was found to contain Ch6S were SCC-BD.

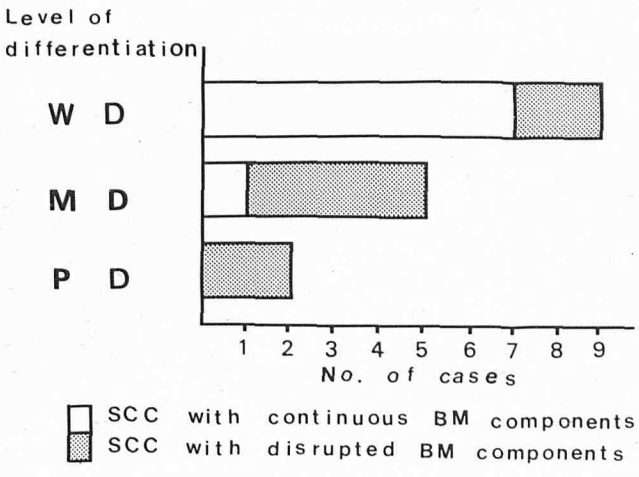

Figure 3. Relationship of continuity of LN, C-4, and HSPG with the level of tumor differentiation. WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

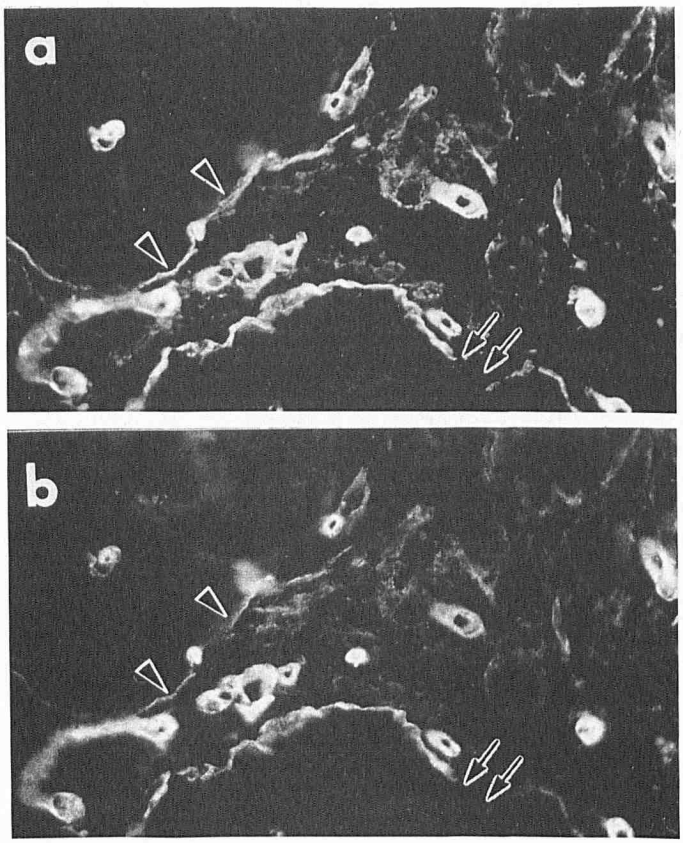

Figure 4. Double immunofluorescent staining of a poorly differentiated SCC-NB with anti-LN and anti-HSPG antibodies. LN (*a*) and HSPG (*b*) are disrupted in the same area of the tumor BM. *Arrows,* disruption of the BM; *arrowhead,* relatively intact tumor BM. (Magnification ×250.)

sections were further incubated with HS47, rinsed again, and then incubated with fluorescein-conjugated rabbit anti-mouse immunoglobulins. The other sections were processed for Ch6S staining as reported by Fine and Couchman [11,12]. Sections were fixed in 4% paraformaldehyde in PBS for 30 min, rinsed in PBS, and then exposed to 0.1 M ammonium chloride in PBS for 15 min. After being rinsed in PBS, the sections were incubated for 30 min at 37°C with chondroitinase ABC (0.1 U/ml in 0.1 M Tris-acetate, pH 7.3, containing 1 mg/ml of bovine serum albumin) or with the same buffer alone as the control.

All the procedures described above were performed at room temperature. Sections were incubated with antibodies for 30 min and then washed in PBS 3 times for 5 min each.

As controls, some sections were incubated with normal rabbit serum instead of the rabbit antiserum or with HK-1 instead of the MoAb.

After further rinsing in PBS, each section was mounted in 90% glycerol in PBS, and then examined under a Zeiss immunofluorescence microscope equipped with appropriate filters.

After these sections were photographed, they were rinsed in PBS, stained with hematoxylin and eosin, and then observed.

Some cryostat sections of SCC-NB were stained with a MoAb that recognized Ch6S (3B3) and a streptavidin-staining kit (BioGenex Laboratories, San Ramon, CA).

RESULTS

In the normal skin, all BM components studied were linearly observed at the DEJ without any defects or fragmentation (Fig 1). The same components were also located on the BM of dermal blood vessels and appendages.

The alterations of the BM components in the 20 SCC studied are summarized in Table I. Among SCC-NB, C-4, LN, and HSPG were consistently detectable in eight cases in the tumor nest BM, although they were irregular and varied in thickness (Fig 2). Seven of the eight were well differentiated SCC and one was a moderately differentiated SCC (Fig 3). Only one of them was metastatic. In the other eight cases of SCC-NB, the BM components were disrupted, and short fragments of these components were observed in some parts of the tumor BM (Fig 4). These BM components were demonstrated by double immunofluorescent staining to have undergone disruption in the same regions of the BM (compare Fig 4*a* with Fig 4*b*). Hematoxylin and eosin staining of these sections showed that disruptions of the BM components were not attributable to damage to the sections, and that these extensive defects were not always associated with collection of inflammatory cells. Only two cases of this group were well-differentiated SCC, and the others were all moderately or poorly differentiated ones (Fig 3). In four of these eight cases, HSPG showed a fibrous pattern in the interstitium around the tumor nests (data not shown). Lymph node metastases were noted in two of these eight cases. Ch6S was absent from the BM of all the SCC-NB examined (Fig 5*b*), although LN and C-4 were detected positively on the BM of the same tumor nests (Fig 5*a*).

Fragmentation of all the components examined, i.e., C-4, LN (Figs 6*a* and 7*a*), HSPG (Fig 6*b*), and Ch6S (Fig 7*b*), was observed in some parts of the BM in three of the four SCC-BD. In these three cases, all the BM components examined had been lost more extensively around the lower tumor nests than the upper ones. Although

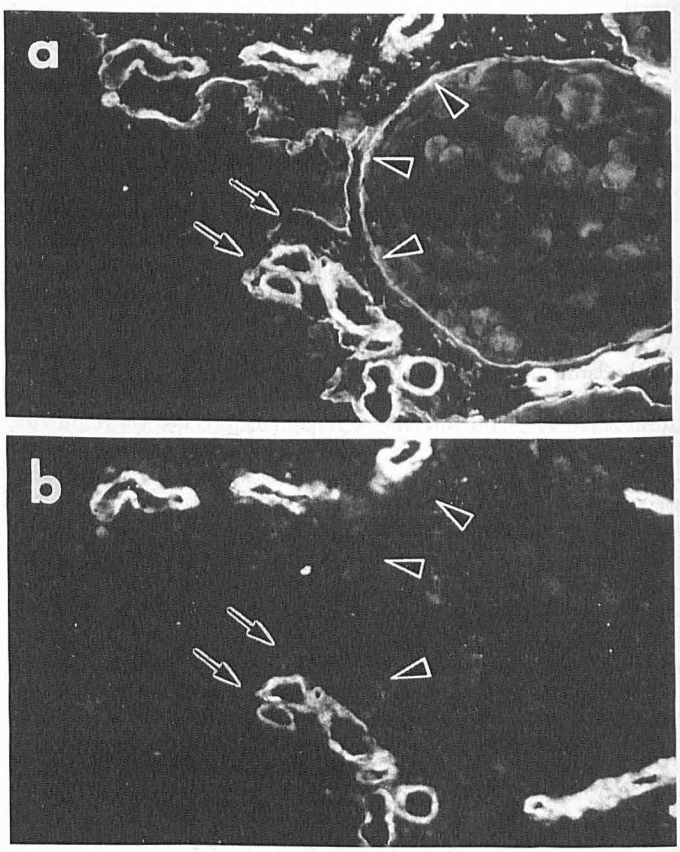

Figure 5. Double immunofluorescent staining of a poorly differentiated SCC-NB with anti-LN and anti-Ch6S antibodies. LN (*a*) is degraded in some areas of the tumor BM. Ch6S (*b*) is absent from the same tumor nests, whereas it exists on capillary BM. *Arrows,* disruption of LN; *arrowheads,* tumor BM. (Magnification ×250.)

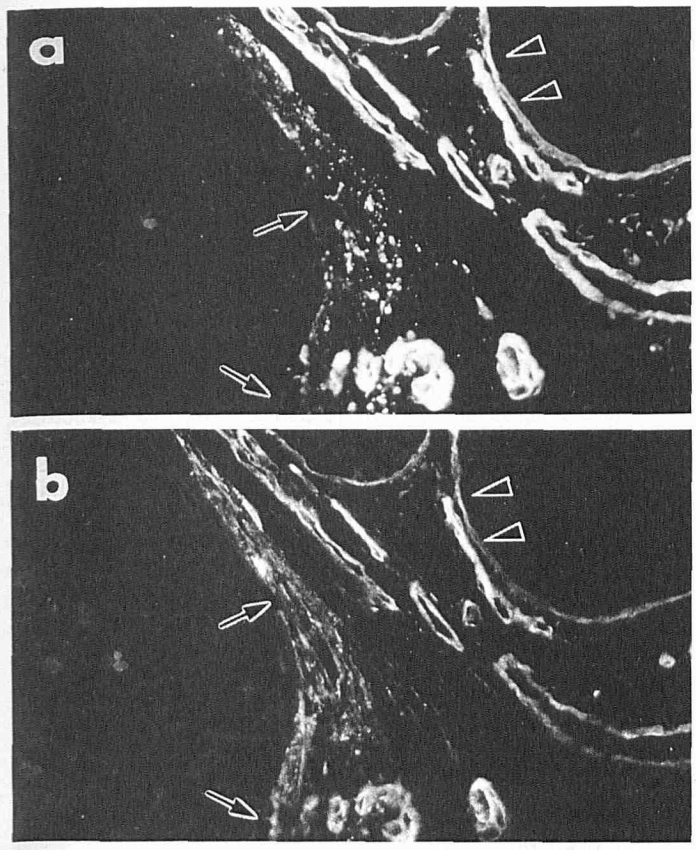

Figure 6. Double immunofluorescent staining of a SCC-BD with anti-LN and anti-HSPG antibodies. LN (*a*) and HSPG (*b*) are more extensively lost in some areas of the BM in the lower nest. *Arrows,* extensively disrupted BM; *arrowheads,* relatively intact BM. (Magnification ×250.)

the C-4, LN, and HSPG in the BM showed no disruption in the remaining case of SCC-BD, Ch6S was not continuous.

No staining was observed of control sections incubated with normal rabbit serum in place of the rabbit antisera, whereas HSPG or Ch6S could be observed as fluorescein staining of the BM area. In addition, there was no staining of the BM of tumor nests in the control sections incubated with HK-1 instead of the MoAb to BM components, whereas C-4 and LN staining were detected. These data indicate that the immunofluorescence method was specific for each BM component. In the capillary BM adjoining tumor nests, Ch6S staining was observed to be as intense as in normal skin (Fig 5*b*), indicating that the loss of immunofluorescence for Ch6S in other regions was not due to technical errors. Immunoperoxidase staining with 3B3 and peroxidase-conjugated streptavidin also failed to show Ch6S in the BM of these SCC (data not shown), indicating that its complete lack was not simply due to the lower sensitivity of immunofluorescence.

DISCUSSION

Because penetration of the epidermal BM by tumors is the first step in the invasion of the dermis and is also required before tumors can enter the circulation to produce distant metastase [1–3], the ability of a tumor to destroy the BM appears to be related to its capacity for invasion [1–3,18] and metastasis [1,2,19]. Some experiments have been performed to study digestion of the BM components by tumors in vitro [19–21]. However, cultured cells do not necessarily maintain the original tumor's characteristics [21], so data obtained from these in vitro experiments cannot be easily extrapolated to evaluate the ability of the original tumors to destroy the BM. In fact, it would seem to be more valuable to study the changes in the BM of actual tumors to evaluate the ability of the tumor to degrade the BM.

Alterations in the BM components of SCC, such as C-4 and LN, have previously been investigated immunohistochemically [4–7]. The reports are contradictory: one claims that the loss of C-4 is associated with tumor invasion [4], whereas the other does not [5–7]. Our present data support the latter contention. However, we found that SCC could be divided into two groups according to the pattern of the BM components C-4, LN, and HSPG. One group consisted of nine cases in which these BM components were continuous, and the other group comprised 11 cases in which these components were extensively disrupted. In SCC-NB, the tumors of the latter group tended to be less keratinized and less differentiated than those of the former group (see results). The loss of BM components by tumors probably reflects either loss of the ability to synthesize and secrete such components or, alternatively, the enhancement of their enzymatic degradation [4–7]. In SCC, there is a correlation between protease activity and the level of cellular differentiation [18]. This suggests that the higher protease activity of less-differentiated SCC could degrade BM components leading to their extensive loss. A positive correlation between the enzymatic activity of tumor-cell lines and their metastatic potential has been demonstrated [19], suggesting that poorly differentiated tumors have a higher potential to metastasize than well-differentiated ones. However, the frequency of metastasis in the SCC showing extensive BM defects was not significantly different from that in the group of tumors with an intact BM. Tumor metastasis is affected not only by tumor's ability to destroy the BM but also by many other factors, such as the immune response of the host [20], the tumor's origin, [22] and the tumor's location [22].

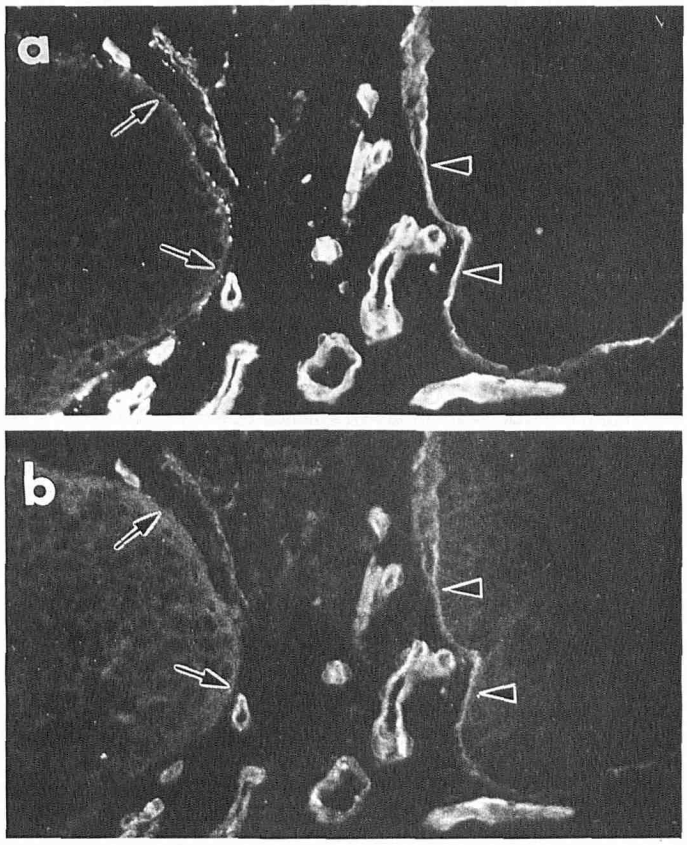

Figure 7. Double immunofluorescent staining of a SCC-BD with anti-LN and anti-Ch6S antibodies. LN (*a*) and Ch6S (*b*) are more extensively lost from the BM of the left nest. *Arrows,* extensively degraded BM; *arrowheads,* relatively intact BM. (Magnification ×250.)

HSPG [9,10] and Ch6S [11] are ultrastructurally located on the basal lamina of the normal DEJ. In addition, the proteoglycan recognized by 3B3 has been suggested to a hybrid containing both HS and Ch6S [12]. However, changes of these proteoglycans in the tumor BM have not previously been investigated. In the present study, double immunofluorescent staining revealed that Ch6S was completely absent from the BM of the SCC-NB, whereas the other BM components were detected in the same tumor nests. A similar absence of Ch6S has been demonstrated in the BM in both dominant and recessive dystrophic epidermolysis bullosa. Because the loss of Ch6S was both specific and complete, it was probably caused by the loss of the ability to synthesize GAG in the course of malignant transformation. Therefore, SCC-NB may have a BM proteoglycan composition from which Ch6S proteoglycan has been selectively depleted. Although it is unknown whether or not the HSPG recognized by HS47 has a core protein identical with that of the hybrid Ch6S/HS proteoglycan described by Fine and Couchman [11,12], this core protein may have an altered structure that lacks Ch6S [12]. In SCC-BD, Ch6S was detected in the BM, indicating that these tumors still preserved the ability to synthesize Ch6S. As with the other BM components, Ch6S was extensively lost around the deeper tumor nests, whereas it could be easily detected around the superficial nests and only showed a few sites of disruptions (see results). It appears that the extensive loss of Ch6S in the deeper tumor nests was due to its enzymatic degradation by tumor cells.

HSPG and chondroitin sulfate proteoglycan interact with fibronectin [8,23,24] and collagen [23–25], suggesting that they play an important role in the deposition of these extracellular matrix components. Therefore, the differences of the proteoglycan composition of the tumor BM noted between SCC-NB and SCC-BD, especially the presence or absence of Ch6S, may produce alterations of the interstitium around the tumor nests that in turn facilitate invasion and metastasis.

REFERENCES

1. Liotta LA, Rao CV, Barsky SH: Tumor invasion and the extracellular matrix. Lab Invest 49:636–649, 1983
2. Liotta LA: Tumor invasion and metastasis. Role of the extracellular matrix: Rhoads memorial award lecture. Cancer Res 46:1–7, 1986
3. Terranova VP, Hujanen ES, Martin GR: Basement membrane and the invasive activity of metastatic tumor cells. J Natl Cancer Inst 77:311–316, 1986
4. Barsky SH, Siegal GP, Jannotta F, Liotta LA: Loss of basement membrane components by invasive tumor but not by their benign counterparts. Lab Invest 49:140–147, 1983
5. McArdle JP, Roff BT, Muller HK, Murphy WH: The basal lamina in basal carcinoma, Bowen's disease, squamous cell carcinoma and keratoacanthoma: an immunoperoxidase study using an antibody to type IV collagen. Pathology 16:67–72, 1984
6. Gusterson BA, Warburton MJ, Mitchell D, Kraft N, Hancock WW: Invading squamous cell carcinoma can retain a basal lamina. An immunohistochemical study using a monoclonal antibody to type IV collagen. Lab Invest 51:82–87, 1984
7. Gusterson BA, Clinton S, Gough G: Studies of early invasive and intraepithelial squamous cell carcinoma using an antibody to type IV collagen. Histopathology 10:161–169, 1986
8. Isemura M, Sato N, Yamaguchi Y, Aikawa J, Munakata H, Hayashi N, Yosizawa Z, Nakamura T, Kubota A, Arakawa M, Hsu C-C: Isolation and characterization of fibronectin-binding proteoglycan carrying both heparan sulfate and dermatan sulfate chains from human placenta. J Biol Chem 262:8926–8933, 1987
9. Caughman SW, Krieg T, Timpl R, Hintner H, Katz SI: Nidogen and heparan sulfate proteoglycan: detection of newly isolated basement membrane components in normal and epidermolysis bullosa skin. J Invest Dermatol 89:547–550, 1987
10. Kazama T, Nakamura T, Isemura M, Sato Y: Immunohistochemical localization of heparan sulfate-containing proteoglycan in normal human skin with monoclonal antibodies: comparison with that of fibronectin. Arch Dermatol Res 281:440–442, 1989
11. Fine J-D, Couchman JR: Chondroitin-6-sulfate containing proteoglycan: a new component of human skin dermoepidermal junction. J Invest Dermatol 90:283–288, 1988
12. Fine J-D, Couchman JR: Chondroitin 6-sulfate proteoglycan but not heparan sulfate proteoglycan is abnormally expressed in skin basement membrane from patients with dominant and recessive dystrophic epidermolysis bullosa. J Invest Dermatol 92:611–616, 1989
13. Hayman EG, Oldberg A, Martin GR, Ruoslahti E: Codistribution of heparan sulfate proteoglycan, laminin, and fibronectin in the extracellular matrix of normal rat kidney cell and their coordinate absence in transformed cells. J Cell Biol 94:28–35, 1982
14. Iozzo RV: Proteoglycans: structure, function, and role in neoplasia. Lab Invest 53:373–396, 1985
15. Timpl R, Rohde H, Robey PG, Rennard SI, Foidart J-M, Martin GR: Laminin-A glycoprotein from basement membranes. J Biol Chem 254:9933–9937, 1979
16. Glanville RW, Rauter A, Fietzek PP: Isolation and characterization of a native placental basement-membrane collagen and its component chains. Eur J Biochem 95:383–389, 1979
17. Hashimoto K, Eto H, Matsumoto M, Hori K: Anti-keratin monoclonal antibodies: production, specificities and applications. J Cutan Pathol 10:529–539, 1983
18. Tsuboi R, Yamaguchi T, Kurita Y, Nakao H, Ogawa H, Ishihara K: Comparison of proteinase activities in squamous cell carcinoma, basal cell epithelioma, and seborrheic keratosis. J Invest Dermatol 90:869–872, 1988
19. Liotta LA, Tryggvason K, Garbisa S, Hart I, Folts CM, Shafie S: Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284:67–68, 1980
20. Eisenbach L, Segal S, Feldman M: Proteolytic enzymes in tumor metastasis: II. Collagenase type IV activity in subcellular fractions of cloned tumor populations. J Natl Cancer Inst 74:87–93, 1985
21. Fidler IJ: Selection of successive tumor lines for metastasis. Nature New Biol 242:148–149, 1973
22. Robson W, Grier N: Squamous cell carcinoma of the body and extremities. In: Andrade R, Gumport SL, Popkin GL, Rees TD (eds.). Cancer of the Skin. Biology—Diagnosis—Management. W. B. Saunders Company, 1976, pp 916–932
23. Ruoslahti E, Engvall E: Complexing of fibronectin, glycosaminoglycans and collagen. Biochim Biophys Acta 631:350–358, 1980
24. Oldberg A, Ruoslahti E: Interactions between chondroitin sulfate proteoglycan, fibronectin, and collagen. J Biol Chem 257:4859–4863, 1982
25. Koda JE, Bernfield M: Heparan sulfate proteoglycans from mouse mammary epithelial cells. Basal extracellular proteoglycan bind specifically to native type I collagen fibrils. J Biol Chem 259:11763–11770, 1984